

# A novel cytostatic form of autophagy in sensitization of non-small cell lung cancer cells to radiation by vitamin D and the vitamin D analog, EB 1089

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**Abbreviations:** ACTB, actin,  $\beta$ ; AMPK, AMP activated protein kinase; AVO, acidic vesicular organelles; ANXA5, annexin A5; ATG5, autophagy related 5; Baf, bafilomycin A<sub>1</sub>; BECN1, Beclin 1, autophagy-related; FACS, fluorescence activating cell sorting; GLB, galactosidase;  $\beta$ ; GFP, green fluorescent protein; H2AFX/H2AX, H2A histone family, member X; MAP1LC3/LC3, microtubule-associated protein 1 light chain 3; NSCLC, non-small cell lung cancer; PI, propidium iodide; SQSTM1, sequestosome 1; TP53, tumor protein p53; VDR, vitamin D (1, 25-dihydroxyvitamin D<sub>3</sub>) receptor; WT, wild-type; LC3 and LC3-II acronyms have been used to indicate the LC3B and LC3BII isoforms respectively.

The standard of care for unresectable lung cancer is chemoradiation. However, therapeutic options are limited and patients are rarely cured. We have previously shown that vitamin D and vitamin D analogs such as EB 1089 can enhance the response to radiation in breast cancer through the promotion of a cytotoxic form of autophagy. In A549 and H460 non-small cell lung cancer (NSCLC) cells, 1,25-D<sub>3</sub> (the hormonally active form of vitamin D) and EB 1089 prolonged the growth arrest induced by radiation alone and suppressed proliferative recovery, which translated to a significant reduction in clonogenic survival. In H838 or H358 NSCLC cells, which lack VDR/vitamin D receptor or functional TP53, respectively, 1,25-D<sub>3</sub> failed to modify the extent of radiation-induced growth arrest or suppress proliferative recovery post-irradiation. Sensitization to radiation in H1299 NSCLC cells was evident only when TP53 was induced in otherwise *tp53*-null H1299 NSCLC cells. Sensitization was not associated with increased DNA damage, decreased DNA repair or an increase in apoptosis, necrosis, or senescence. Instead sensitization appeared to be a consequence of the conversion of the cytoprotective autophagy induced by radiation alone to a novel cytostatic form of autophagy by the combination of 1,25-D<sub>3</sub> or EB 1089 with radiation. While both pharmacological and genetic suppression of autophagy or inhibition of AMPK phosphorylation sensitized the NSCLC cells to radiation alone, inhibition of the cytostatic autophagy induced by the combination treatment reversed sensitization. Evidence for selectivity was provided by lack of radiosensitization in normal human bronchial cells and cardiomyocytes. Taken together, these studies have identified a unique cytostatic function of autophagy that appears to be mediated by VDR, TP53, and possibly AMPK in the promotion of an enhanced response to radiation by 1,25-D<sub>3</sub> and EB 1089 in NSCLC.

## Introduction

Lung cancer is the leading cause of cancer-related death in the United States,<sup>1</sup> accounting for 29% of male deaths and 26% of female deaths.<sup>2</sup> Lung cancer has a poor prognosis with a median survival rate of less than 12 mo.<sup>3</sup> Lung cancer is frequently unresectable and the current available therapies such as radiation and chemotherapy, while generally increasing patient survival, are

frequently only palliative.<sup>3,4</sup> Therefore, there is a pressing need for the development of new therapies that are more effective and selective than what is currently available for the treatment of lung cancer patients.

The standard of care for lung cancer is generally radiation in combination with chemotherapy to maximize its impact.<sup>5,6</sup> A limitation of radiotherapy is tumor cell resistance either by over-activation of compensatory cell growth pathways or DNA repair

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pathways; this can often result in disease recurrence.<sup>7</sup> One potential approach for overcoming these limitations could be to combine radiation treatment with vitamin D or vitamin D analogs (which tend to be less hypercalcemic) to enhance the response to radiation therapy.<sup>8-10</sup> Vitamin D is a steroid hormone that plays an important role in maintaining calcium homeostasis.<sup>11</sup> Vitamin D has also been shown to have antiproliferative and antitumorigenic effects.<sup>11,12</sup> A number of Vitamin D analogs, some of which are being used in clinical trials, have been developed that have also shown antitumor effects.<sup>13</sup>

Vitamin D exerts its biological activity by binding to VDR (vitamin D [1,25-dihydroxyvitamin D<sub>3</sub>] receptor), which is a member of the steroid-thyroid-retinoid gene super family.<sup>14</sup> The VDR acts as a transcriptional regulator, which is activated by binding of the ligand, 1,25-D<sub>3</sub>, which leads to formation of a heterodimer between VDR and RXR (retinoid X receptor). This complex recognizes and binds to vitamin D responsive elements, which further leads to recruitment of nuclear receptor coactivator proteins and VDR-interacting proteins. This recruitment causes an increase in VDR transcriptional activation.<sup>14,15</sup> Clinically it has been shown that lung cancers express VDRs, indicating that they would likely be responsive to vitamin D.<sup>14-16</sup> The literature suggests that vitamin D deficiency may contribute to cancer and high circulating levels of 25 (OH)-D<sub>3</sub>, which is an immediate precursor of calcitriol (1,25-D<sub>3</sub>), have been associated with prolonged survival of NSCLC patients.<sup>17</sup> Studies have also shown a link between improved survival rate in lung cancer patients and high level of expression of VDR.<sup>14</sup> Finally, previous studies in our laboratory have demonstrated the capacity of 1,25-D<sub>3</sub> as well as the vitamin D analog, EB 1089, to enhance the response to radiation in breast tumor cells, both in cell culture and in tumor bearing mice.<sup>8-10,18,19</sup>

The current work defines a new mechanism whereby 1,25-D<sub>3</sub> and the vitamin D analog EB 1089 enhance sensitivity to radiation in NSCLC cells. We observed that autophagy that is induced by radiation in combination with either 1,25-D<sub>3</sub> or EB 1089 has a cytostatic function that translates into an enhanced reduction of clonogenic survival. Further, we observed the apparent conversion of the cytoprotective form of autophagy induced by radiation alone to a cytostatic form of autophagy by radiation in combination with +1,25-D<sub>3</sub> or EB 1089.

## Results

### Influence of 1,25-D<sub>3</sub> and the vitamin D analog EB 1089 on sensitivity to radiation in non-small cell lung cancer cells

Figure S1A shows expression levels for VDR, which is generally considered to be required for vitamin D to affect cell function,<sup>20</sup> in a number of NSCLC cell lines. High levels of the receptor are evident in the A549, H460, and H358 cell lines and the receptor is also clearly expressed in the H1299 and H520 cell lines but is barely detectable in the H838 or H2030 cells.

Figure 1A and B present clonogenic survival studies performed at different doses of radiation alone (varying from 2Gy to 6Gy) and in combination with either the vitamin D analog, EB

1089, or 1,25-D<sub>3</sub> in the H460 and A549 cells, respectively. The inclusion of either 1,25-D<sub>3</sub> or EB 1089 resulted in a significantly greater loss of clonogenicity at every radiation dose, with sensitization factors of between 1.4 and 2.0. Temporal response studies assessing cell viability in Figure 1C and D corroborated the results of the clonogenic survival study. While a single dose of 6Gy results in transient growth inhibition that is followed by proliferative recovery, cell growth inhibition is more pronounced and proliferative recovery is significantly impaired with the combination treatment of radiation + 1,25-D<sub>3</sub> or EB 1089. These results differ from our findings in breast tumor cells where the combination treatment resulted in clear evidence of cell killing (a time-dependent decline in viable cell number).<sup>18,19,21</sup>

Clonogenic survival studies shown in Figure S1B and C indicate that neither 1,25-D<sub>3</sub> (the active form of vitamin D) nor EB 1089 (a synthetic analog of vitamin D) alone, at concentrations of 10 nM, 50 nM and 100 nM, significantly interfered with growth of the A549 or H460 NSCLC cells. Similarly, cell viability studies shown in Figure S1D and E indicated that neither 1,25-D<sub>3</sub> nor EB 1089 inhibited growth or survival of H460 and A549 cells at 100 nM, the concentration used in the current work as well as our previous studies in breast cancer. The lack of effect of these agents alone establishes that the sensitization effects observed are not simply the consequence of additive antiproliferative or cytotoxic interactions. Furthermore, treatment with 1,25-D<sub>3</sub> and EB 1089 had an essentially identical impact in terms of tumor cell sensitization (sensitization by 1,25-D<sub>3</sub> and EB 1089 was significant for both cell lines; this becomes apparent when the data is plotted on an expanded scale). Consequently, while the bulk of studies in A549 cells were performed using 1,25-D<sub>3</sub>, studies in H460 cells were performed using EB 1089, which tends to be more stable for prolonged periods in solution. Additionally, in terms of potential clinical utility, EB 1089 has reduced calcemic effects.<sup>22</sup>

### 1,25-D<sub>3</sub> and the vitamin D analog EB 1089 fail to increase the extent of DNA damage induced by IR or interfere with DNA repair in non-small cell lung cancer cells

In our previous studies with breast tumor cells, we have found no evidence for increased DNA damage or decreased DNA repair associated with sensitization to radiation by 1,25-D<sub>3</sub>/EB 1089.<sup>8,19,21</sup> Figure S2 presents studies in which DNA damage was assessed by measuring levels of phosphorylated H2AFX/γH2AX in response to radiation and radiation + EB 1089 in the H460 cells. DNA damage was observed within 1 h after irradiation and declined thereafter, but neither the initial extent of damage nor the rate of decline, indicative of DNA repair, was affected by the presence of the EB 1089. It is also worth noting that there was no effect of EB 1089 alone, consistent with the lack of effect on cell growth.

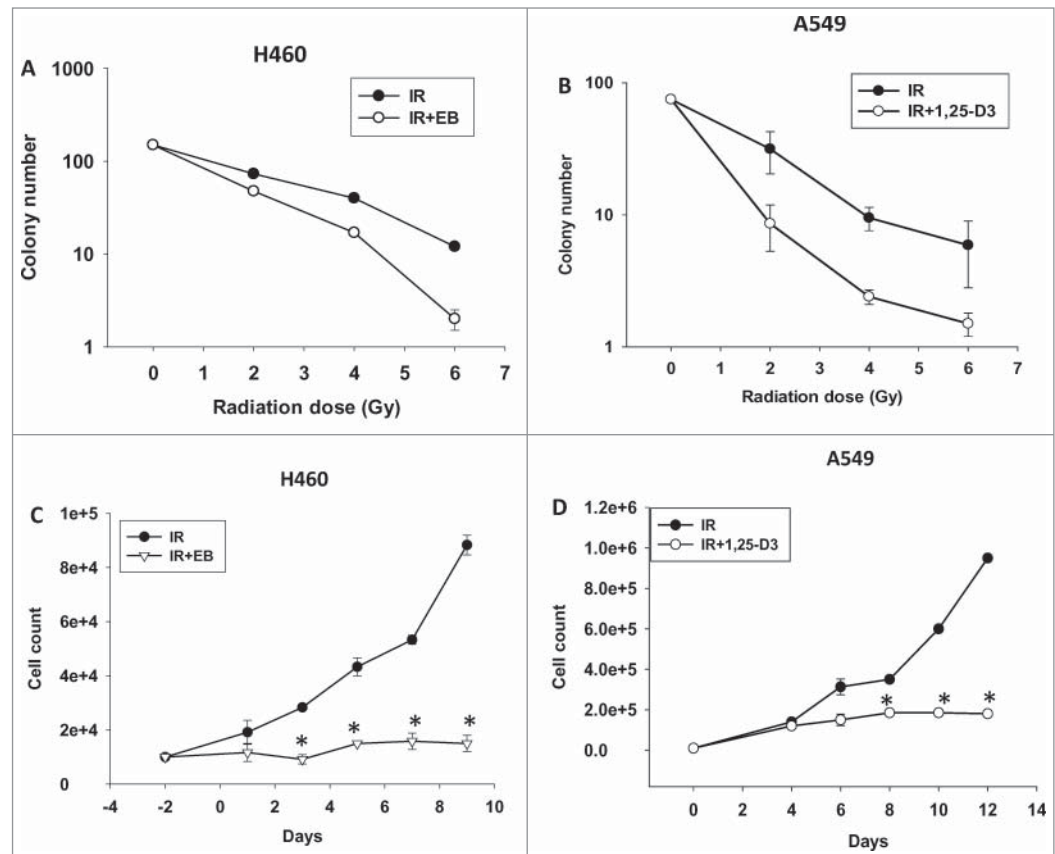
### 1,25-D<sub>3</sub> or EB 1089 fail to increase the extent of senescence induction by radiation in NSCLC cells

In studies that were performed prior to recognition of the potential role of autophagy in the response to radiation, we

demonstrated that the primary response to radiation is senescence,<sup>23,24</sup> in the absence of significant apoptosis, in cells expressing wild-type TP53/TRP53/p53 (note that the mouse nomenclature is TRP53, but we will use TP53 for either the human and mouse genes/proteins for simplicity). Since the temporal cell viability studies in **Figure 1** indicated that the cells were undergoing primarily growth arrest, we evaluated whether the extent of senescence might have been increased for the combination treatments. **Figure S3A and B** present GLB (galactosidase,  $\beta$ )-staining images in A549 and H460 NSCLC cell lines, which indicates that both radiation treatment alone and the combination of radiation with 1,25-D<sub>3</sub> or EB 1089 leads to an increase in GLB staining and alterations in morphology indicative of senescence. **Figure S3C and D** provide quantification of the time-dependent increase in senescence induced by radiation and the combination treatments in A549 (left panel) and H460 (right panel) cell lines. These studies fail to demonstrate any significant differences between radiation treatment alone and the combination of radiation + 1,25-D<sub>3</sub> or EB 1089 in terms of the extent of senescence.

#### Minimal induction of apoptosis or necrosis by radiation and radiation + 1,25-D<sub>3</sub> or EB 1089 in NSCLC cells

Although the primary responses observed either with radiation alone or radiation + EB 1089 or radiation + 1,25-D<sub>3</sub> appeared to be growth arrest, it was formally possible that a fraction of the cell population might be undergoing apoptosis (or necrosis). Apoptosis and necrosis were evaluated by ANXA5 and PtdIns staining and flow cytometry. **Figures S4A and Figure S4B** show that there was minimal induction of apoptosis or necrosis (data for necrosis not shown) by radiation and radiation + 1,25-D<sub>3</sub> or EB 1089 treatments in A549 and H460 cell lines. This outcome was further confirmed by the lack of PARP (poly ADP ribose polymerase) cleavage where staurosporine was used as a positive control (data not shown).

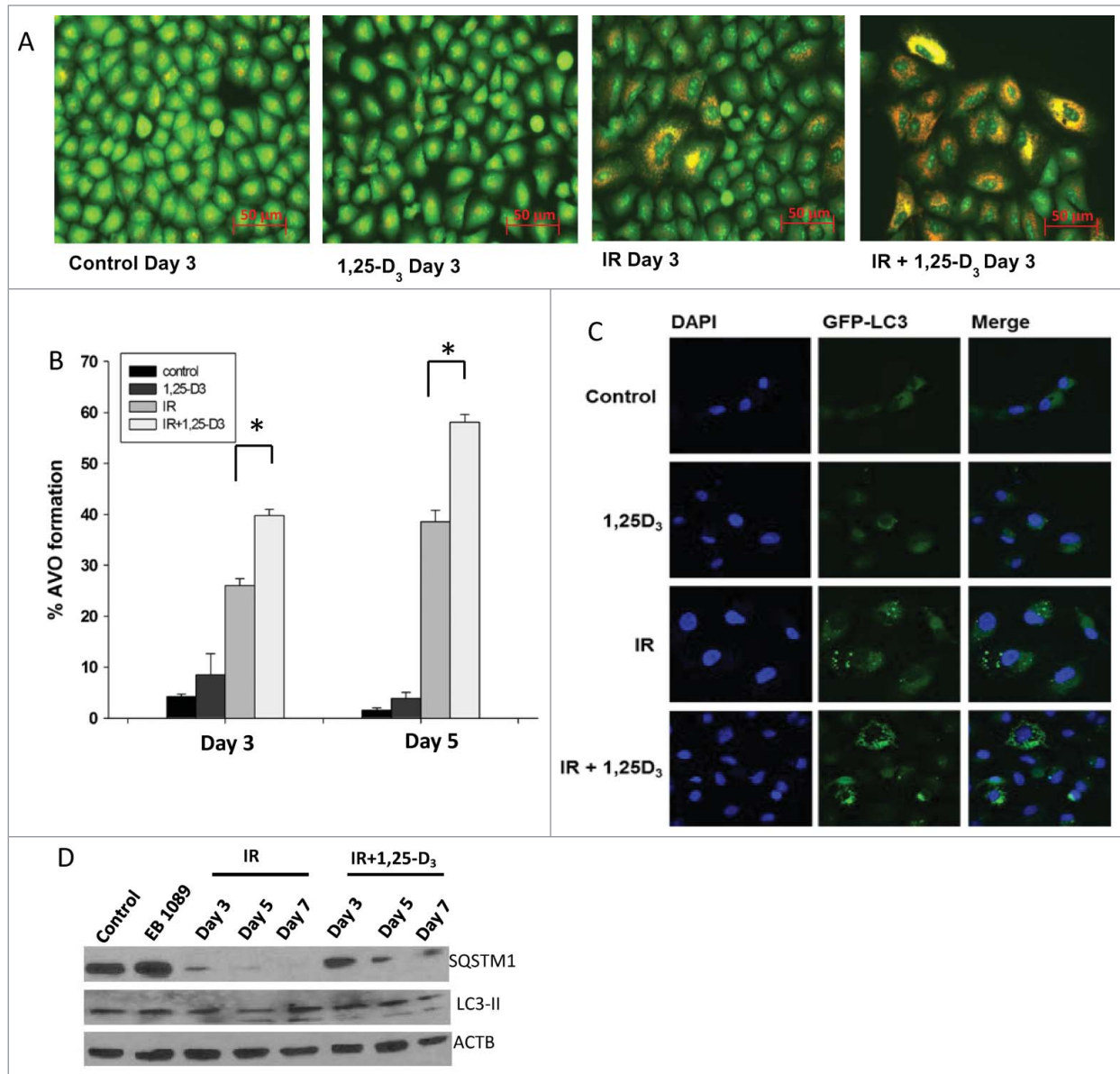


**Figure 1.** Radiosensitization of NSCLC cells. H460 (A) and A549 (B) NSCLC cells were exposed to various doses of ionizing radiation (IR) alone or in combination with 100 nM EB 1089 or 100 nM 1,25-D<sub>3</sub> and clonogenic survival was assessed after 14 d. H460 cells (C) or A549 cells (D) both of which are wt TP53 and VDR<sup>+</sup>, were exposed to radiation (IR, 6 Gy) alone or in combination with either 100 nM EB 1089 or 1,25-D<sub>3</sub>. Viable cell number was determined at the indicated days following radiation exposure (n = 3, mean ± SE, \*P < 0.05).

#### Increased sensitivity to radiation is associated with a novel form of autophagy

We have previously reported quite extensively on the capacity of 1,25-D<sub>3</sub> and vitamin D analogs to sensitize breast tumor cells to radiation.<sup>8-10,18,19,21</sup> In recent work, we have confirmed that this sensitization, a decline in viable cell number, occurs through the promotion of autophagy based on reversal of sensitization through either pharmacological or genetic interference with autophagy.<sup>18,19</sup> The studies presented below indicate that sensitization in the NSCLC cells is related to conversion of the cytoprotective autophagy induced by radiation alone to what we have termed “cytostatic autophagy” when the combination of radiation and radiation + 1,25-D<sub>3</sub> or EB 1089 is used.

Studies in the A549 cells exposed to either radiation alone or radiation + 1,25-D<sub>3</sub> indicated that radiation alone promotes autophagy, based on acridine orange staining and formation of autophagic puncta (**Fig. 2A**). **Figure 2B** indicates that the extent of autophagic vesicle formation (as quantified by flow cytometry) was significantly increased by the combination of radiation and 1,25-D<sub>3</sub> in the A549 cells. GFP-LC3 puncta formation shown in **Figure 2C** further corroborates the flow cytometry data indicative of the promotion of autophagy.

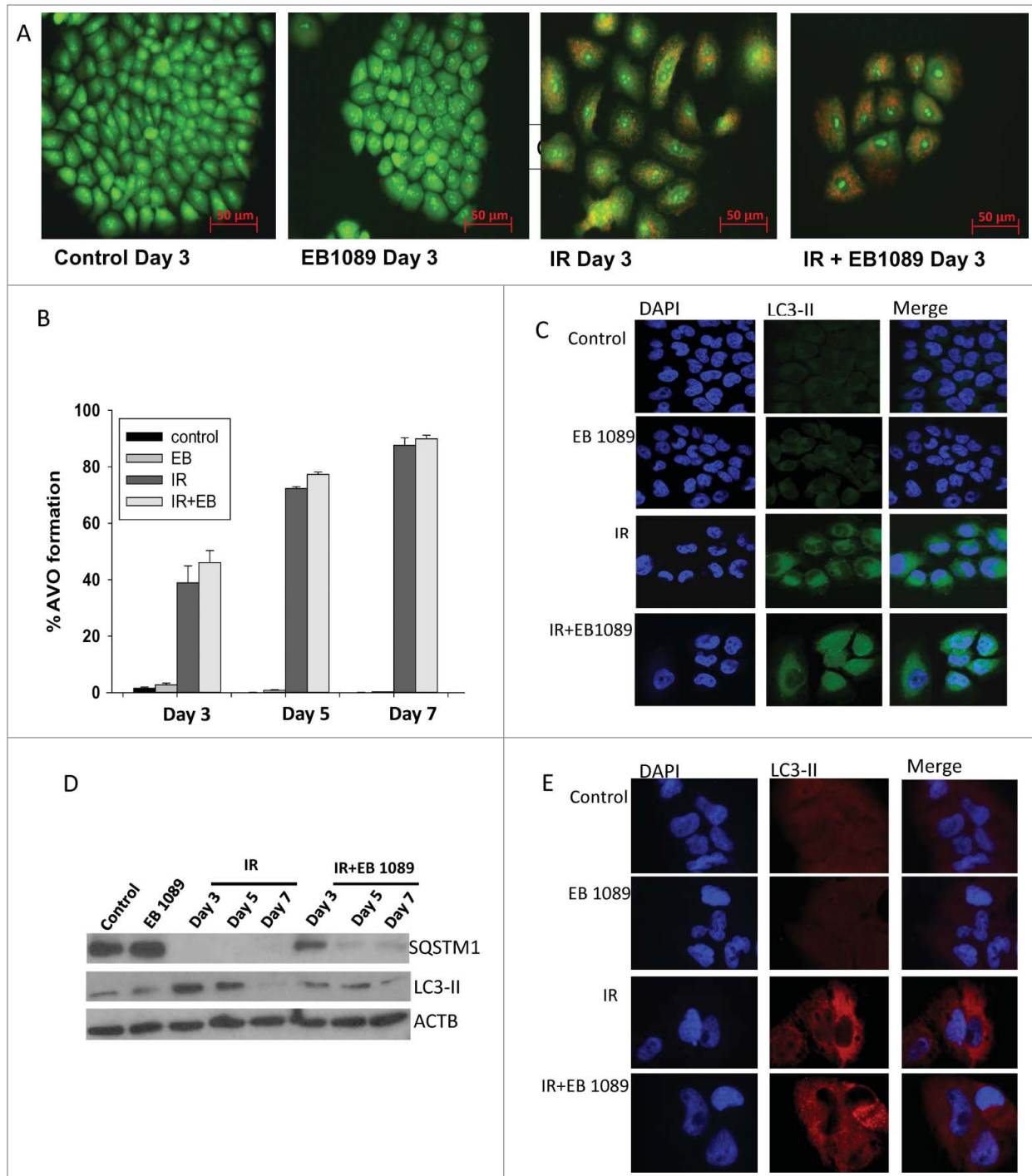


**Figure 2.** Autophagy in A549 NSCLC cells. (A) Induction of autophagy in A549 cells by radiation (IR, 6 Gy) alone or radiation+1,25-D<sub>3</sub> by acridine orange staining. (B) Quantification of autophagy by flow cytometry in A549 cells (n = 3, mean ± SE, \*P < 0.05). (C) A549 cells exposed to vehicle control, 100 nM 1,25-D<sub>3</sub>, radiation (IR, 6Gy), or 100 nM 1,25-D<sub>3</sub> in combination with radiation. GFP-LC3 puncta indicate induction of autophagy (all images were taken at the same magnification). (D) Assessment of autophagic proteins, SQSTM1, and LC3-II in response to different treatment conditions by protein gel blots.

Figure 3A and B show autophagic vesicle formation by acridine orange staining and quantification by flow cytometry in the H460 cells; GFP-LC3 puncta formation further indicative of autophagy is shown in Figure 3C and endogenous LC3-II levels assessed using confocal microscopy are shown in Figure 3E. However, as shown in Figure 3B, the extent of autophagy was not significantly different for radiation alone and EB 1089 + radiation in the H460 cells. Consequently, despite the evidence for increased autophagy for the combination treatment in the A549 cells, these findings in H460 cells argue *against the simple explanation that sensitization might be caused solely by an increase in the extent of autophagy.*

Experiments were also performed to determine whether the autophagy observed reflected autophagic flux, where autophagy proceeds to completion in terms of the degradation of select protein or organelle substrates.<sup>25</sup> Figure 2D and Figure 3D demonstrate that autophagic flux markers SQSTM1/p62 and LC3-II were degraded both with radiation alone and the combination treatment in A549 and H460 cells. However, there was no apparent difference in the extent or time course of degradation for radiation alone versus the combination treatment with either 1,25-D<sub>3</sub> or EB 1089. Furthermore, as shown in Figure 4A and B, respectively, the autophagy inhibitors bafilomycin A<sub>1</sub> (Baf) and chloroquine interfered with SQSTM1 degradation by



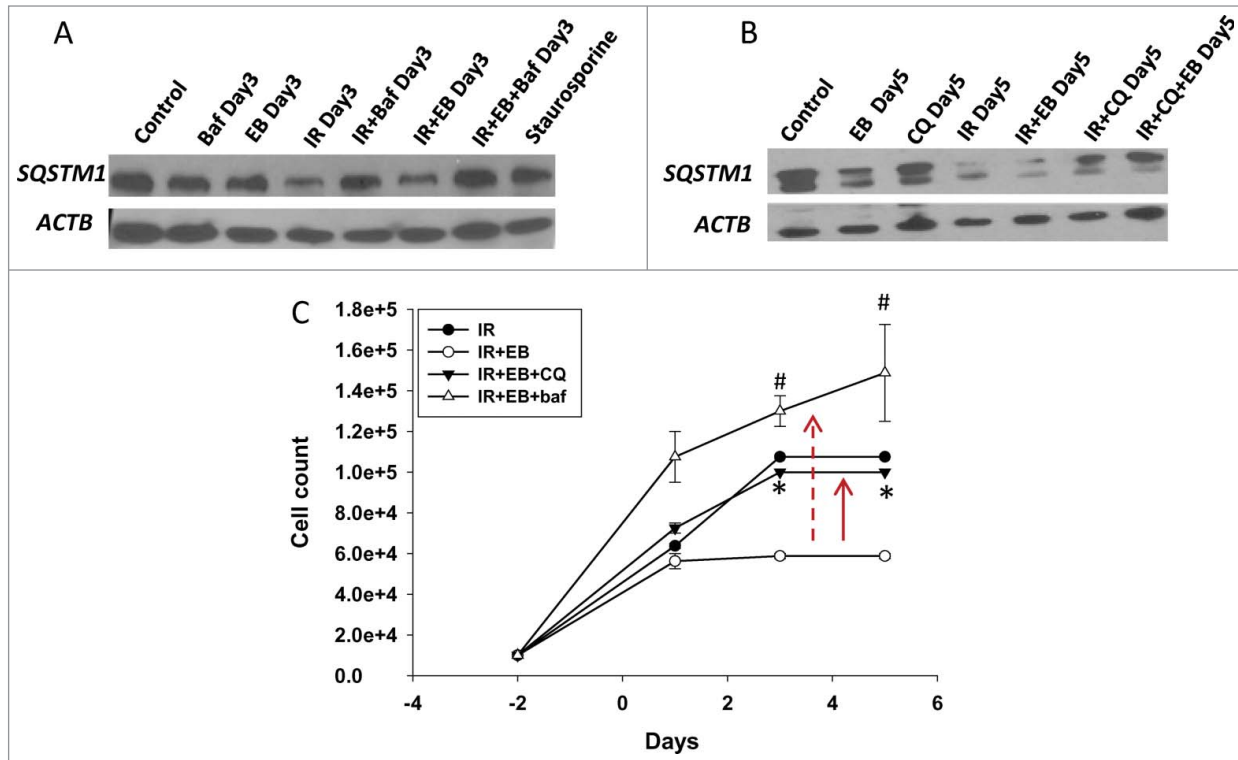


**Figure 3.** Autophagy in H460 NSCLC cells. (A) Induction of autophagy in H460 cells by radiation alone or the combination of radiation+ EB 1089. (B) Quantification of acidic vesicle formation in H460 cells by flow cytometry. (n = 3, mean ± SE) (C) GFP-LC3 puncta formation indicative of the induction of autophagy in H460 cells (all images were taken at same magnification). (D) Assessment of autophagic flux markers, SQSTM1 and LC3-II in response to different treatment conditions by protein gel blots. (E) Endogenous LC3-II foci formation was determined by confocal microscopy. Nuclei were stained with DAPI. Images were taken on d 3 at the same magnification.

radiation alone as well as the combination of radiation with EB 1089, consistent with both treatments promoting autophagic flux.

It has been established by a number of laboratories, including our own, that radiation-induced autophagy frequently has a

cytoprotective function.<sup>18,19</sup> Recent studies by Kroemer's lab have shown this to be the case in the A549 and H460 cells by demonstrating that inhibition of radiation-induced autophagy increases sensitivity to radiation<sup>26</sup> and this has been confirmed in our current work. **Figure S5A and B** show that radiation



**Figure 4.** Evidence for cytosstatic autophagy by pharmacological inhibition. (A, B) Western blot indicating that pharmacological inhibitors Baf (100 nM, panel A) and chloroquine (10  $\mu$ M; panel B) inhibit autophagy by blocking degradation of SQSTM1 in lysates collected from H460 cells. (C) H460 NSCLC cells were exposed to EB 1089 (100 nM) in combination with 6 Gy radiation in the presence of chloroquine (CQ, 10  $\mu$ M) or Baf (100 nM) and cell viability was monitored for a period of 6 d ( $n = 3$ , mean  $\pm$  SE).

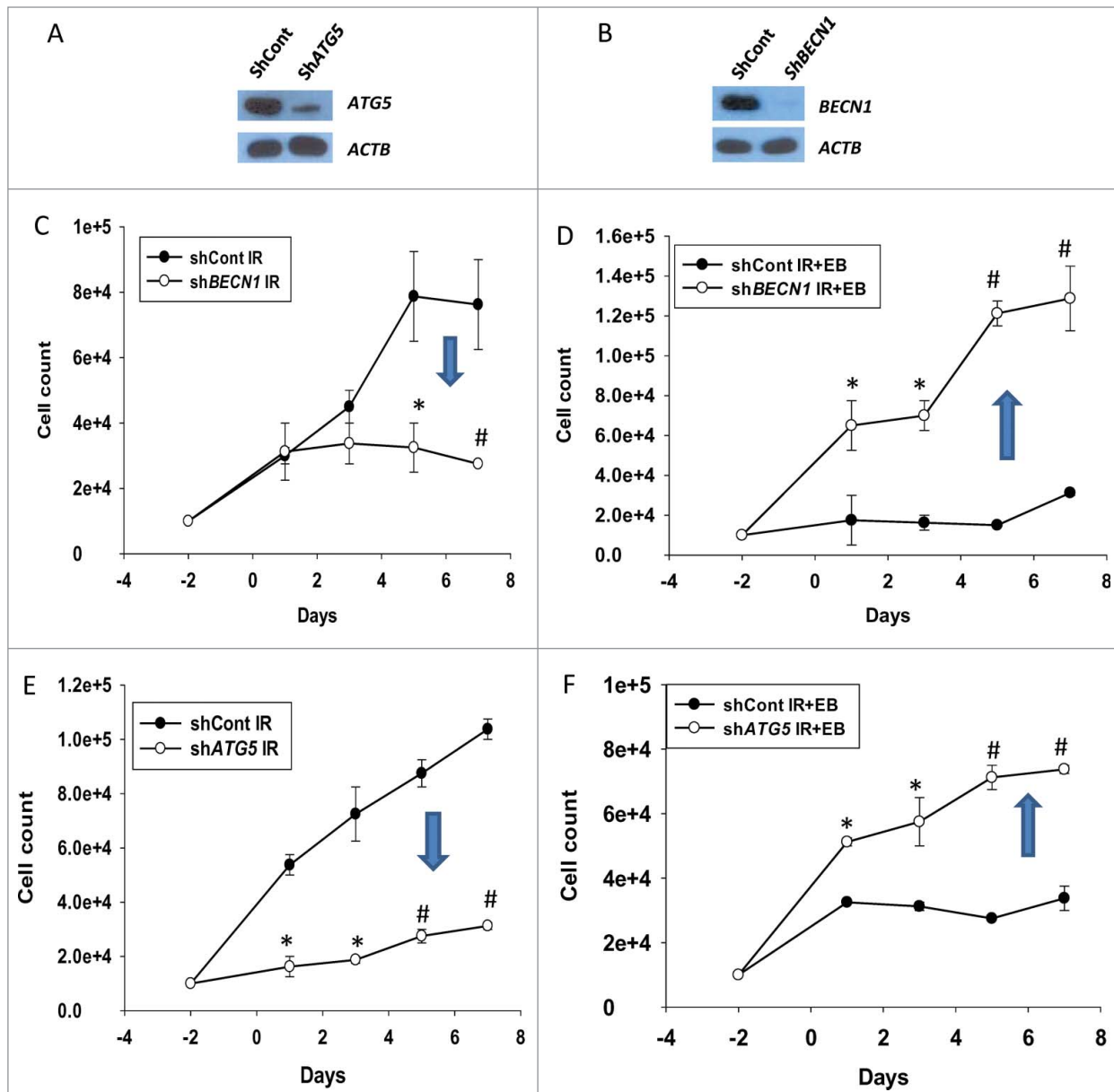
sensitivity increases (downward arrow), when autophagy is blocked by chloroquine in both A549 and H460 cell lines, respectively, consistent with the cytoprotective function of autophagy in response to radiation alone. Furthermore, **Figure S5C and D** demonstrate that blocking cytoprotective autophagy by either chloroquine or Baf led to a modest (but not statistically significant) increase in apoptosis. In contrast, no increase in apoptosis was observed when autophagy was inhibited for the combination treatment. We therefore hypothesized that exposure of the NSCLC cells to 1,25-D<sub>3</sub> or EB 1089 could be altering the *function* of the autophagy, converting the cytoprotective autophagy induced by radiation to what we have termed cytosstatic autophagy, based on the temporal responses to treatment observed in **Figure 1C and D**.

To investigate whether an altered function of autophagy might be responsible for the apparent increase in sensitivity to radiation, we first determined whether interference with autophagy could reverse the impact of radiation + EB 1089, using chloroquine and Baf, both well-characterized pharmacological inhibitors of the late stages of autophagy.<sup>25</sup> As indicated above, **Figure 4A and B** present western blots indicating that both inhibitors by themselves and in combination with radiation and EB 1089, lead to an accumulation of SQSTM1, indicative of the inhibition of autophagic flux.<sup>25</sup> As shown in **Figure 4C**, both chloroquine and Baf, when used to block autophagy, reversed the

radiosensitization effects of EB 1089 (upward arrows). This indicates that what we have termed a cytosstatic form of autophagy (based on the temporal response studies presented in **Fig. 1C and D**) is likely to be the mode of radiosensitization with the combination treatment.

The conclusion that the cytoprotective autophagy induced by radiation has been converted to cytosstatic autophagy by the inclusion of 1,25-D<sub>3</sub> or EB 1089 is strongly supported by complementary genetic silencing studies, where the autophagy-related genes *BECN1* and *ATG5* were knocked down utilizing shRNA, as shown in the immunoblots in **Figure 5A and B**. **Figure S6** confirms successful inhibition of autophagy, based on the reduction in the extent of autophagic vesicle formation, by genetic silencing of *BECN1* and *ATG5*. (It should also be noted that the increased cell size that is a component of autophagy induction is not observed in cells where the autophagy genes have been silenced). The temporal and cell viability studies in **Figure 5D and F** indicate that as was the case with pharmacological inhibition of autophagy, radiosensitization by EB 1089 is *reversed* when cytosstatic autophagy is genetically suppressed. In dramatic contrast, **Figure 5C and E** demonstrate that when cytoprotective autophagy is genetically suppressed, tumor cell sensitivity to radiation is *increased*.

It is important to note that the sensitization to radiation that occurs when the cytoprotective autophagy induced by radiation is



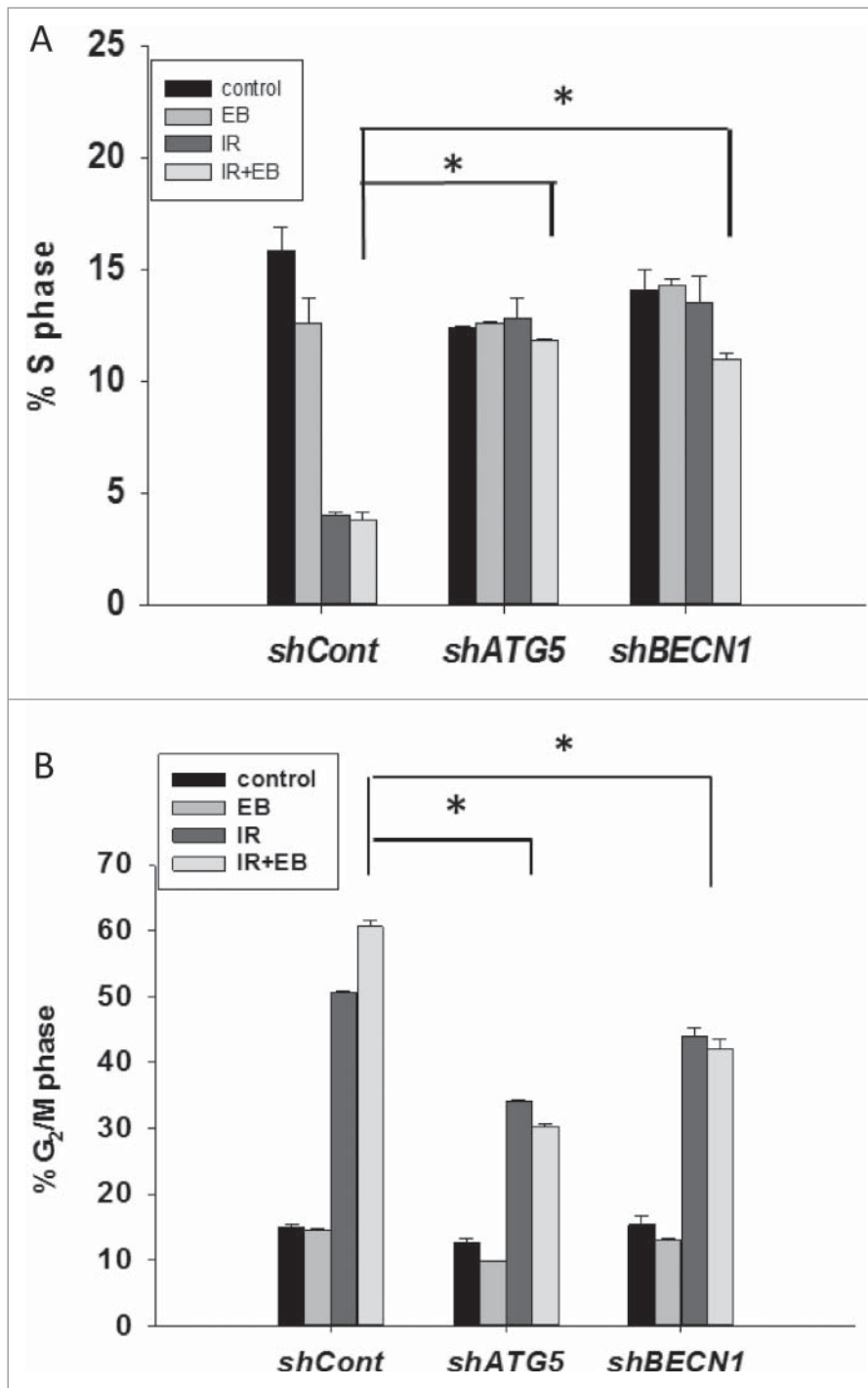
**Figure 5.** Cytoprotective and cytostatic autophagy (genetic silencing). (A) Silencing of *ATG5* and (B) *BECN1* in H460 NSCLC cells. ((C) and D) Effect of *BECN1* silencing on response to radiation alone or EB 1089 + radiation was assessed by cell viability studies (n = 3, mean ± SE, \*P < 0.05, #P < 0.001). ((E) and F) Effect of *ATG5* silencing on response to radiation alone or EB 1089 + radiation was assessed by cell viability studies (n = 3, mean ± SE, \*P < 0.05, #P < 0.001).

inhibited (Fig. 5C and 5E) is no longer evident in the cells exposed to EB 1089 + radiation; otherwise, it would be difficult to reverse the sensitization induced by EB 1089, a problem we encountered in our studies in breast tumor cells.<sup>18,19</sup> This is the basis for our conclusion that the *cytoprotective* form of autophagy induced by radiation has been *converted* to a *cytostatic* form.

#### The effects of radiation and EB 1089 + radiation are associated with cell cycle arrest

As shown in the temporal response studies presented in Figure 1, the primary effects of radiation as well as radiation in combination

with either 1,25-D<sub>3</sub> or EB 1089 were to arrest cell growth without evidence for cell killing in either condition. To confirm these observations, cell cycle analysis was performed in H460 cells exposed to radiation alone and EB 1089 + radiation with and without knock-down of the autophagy genes *ATG5* and *BECN1*. As shown in Figure 6A and B, radiation alone as well as EB 1089 + radiation reduced the S phase population and increased the G<sub>2</sub>/M phase population. Effects of EB 1089 alone on cell cycle distribution were not significant, again consistent with its lack of effect on cell growth. As would be expected, inhibition of autophagy by genetic silencing reversed the G<sub>2</sub>/M associated growth arrest.



**Figure 6.** Cell cycle analysis. Cell cycle analysis was performed in H460 (shcont, shATG5, and shBECN1) cells. (A) Percentage of cells undergoing S-phase is shown (n = 2, mean ± SE, \*P < 0.05; shcont IR+EB 1089 compared to shATG5 IR+EB 1089 and shCont IR+EB 1089 is compared to shBECN1 IR+EB 1089). (B) Percentage of cells arresting in G<sub>2</sub>/M phase is shown. n = 2, mean ± SE, \*P < 0.05; shCont IR+EB 1089 compared to shATG5 IR+EB 1089, and shCont IR+EB 1089 is compared to shBECN1 IR+EB 1089.

#### Requirement for functional TP53 and VDR in sensitization of NSCLC cells

In our previous work with breast tumor cells, we identified a fundamental requirement for functional TP53 in

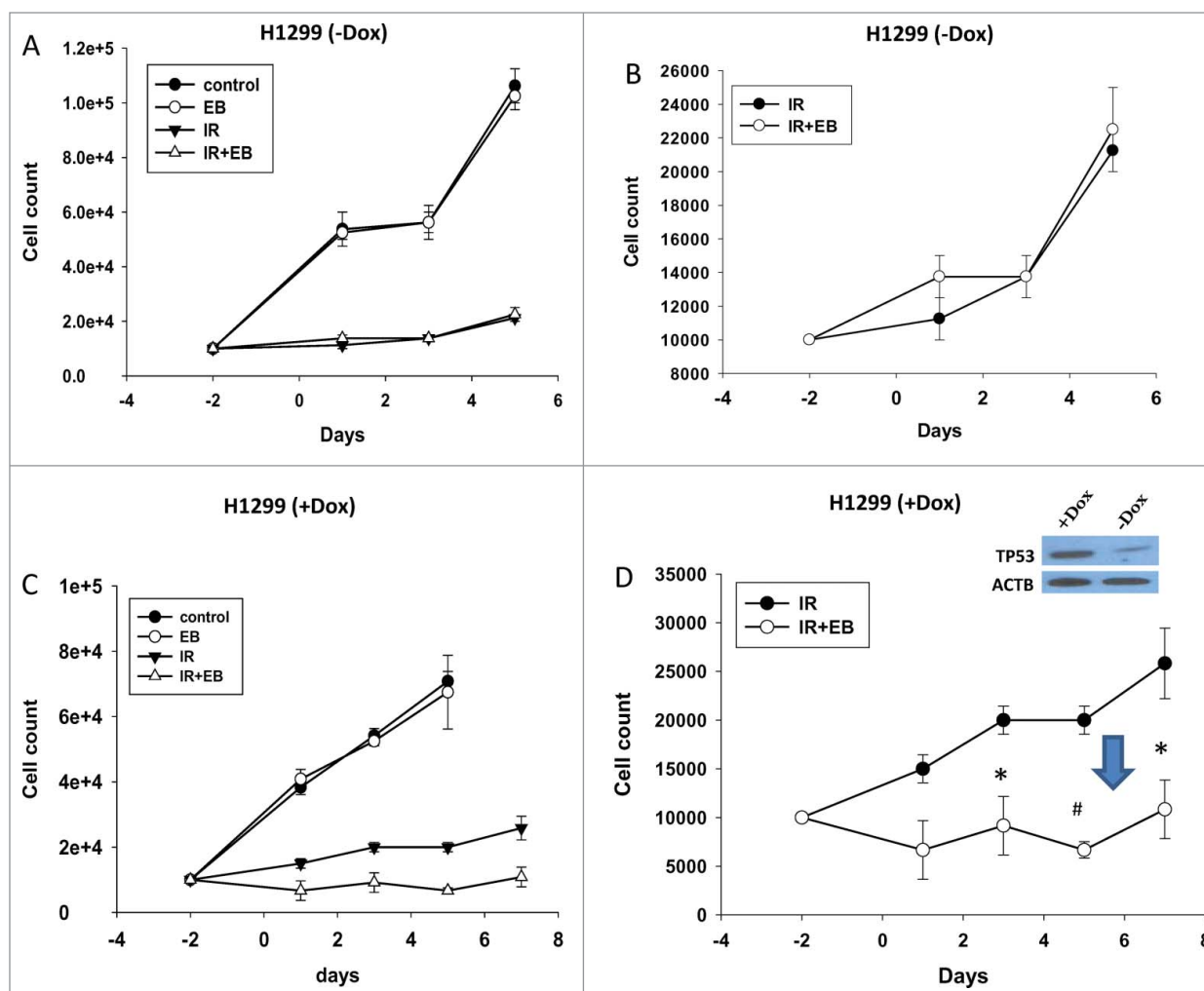
radiosensitization by 1,25-D<sub>3</sub> or EB 1089.<sup>8,18,19</sup> Both A549 and H460 cells are known to express wild-type TP53.<sup>27,28</sup> However the combination treatment of radiation +EB 1089 failed to increase sensitivity to radiation in the H358 NSCLC cell line (Fig. S7A), which has mutant TP53, despite the very high expression of the VDR (Fig. S1A). To establish the importance of the requirement for VDR in enhancement of the response to radiation, similar combination studies were performed in H838 cells, which lack VDR (Fig. S1A) but are wild type in TP53.<sup>29</sup> Figure S7B indicates that these cells did not show any enhancement in the response to the combination treatment of radiation + 1,25-D<sub>3</sub> compared to radiation treatment alone.

To confirm this requirement for functional TP53 in radiosensitization, we demonstrate (Fig. 7A and B, where 7B is an expanded scale for IR and IR+EB 1089) that sensitization to radiation by EB 1089 also fails to occur in the H1299 NSCLC cell line, which is *tp53*-null,<sup>30</sup> but expresses the VDR (Fig. S1A). In dramatic contrast, Figure 7C and D (7D is an expanded scale for IR + EB 1089) indicate that H1299 cells could be radiosensitized by EB 1089 (downward arrow) when TP53 was expressed under the influence of doxycycline (induction of TP53 shown in figure inset).

To confirm and extend these observations in the H1299 cells, we investigated the effects of both pharmacological and genetic autophagy inhibition in both the *tp53*-null and inducible cell lines. As shown in Figure S8A and B, both *ATG5* and *BECN1* were successfully silenced for *tp53*-null and *tp53*-inducible H1299 cells. Figure S8C shows the reduction in autophagic vesicle formation when *ATG5* and *BECN1* were knocked down while Figure S8D shows that chloroquine effectively promoted accumulation of LC3-II, which is indicative of interference with the late stage of autophagy. Figures 8B, D and F indicate that in H1299 cells with induced TP53, sensitization by the combination treatment was reversed when

autophagy was blocked. In quite dramatic contrast, blocking autophagy in the *tp53*-null H1299 parental cells had minimal effect (chloroquine) or no effect (genetic silencing of *ATG5* or *BECN1*) on tumor cell sensitivity to the combination





**Figure 7.** Functional TP53 is required for sensitization of NSCLC cells to radiation. ((A)and B) *tp53*-null H1299 cells and ((C)and D) H1299 cells with TP53 induced by doxycycline (inset) were exposed to radiation (IR, 6 Gy) or EB 1089 in combination with radiation and cell viability was monitored for a period of 5 to 7 d. n = 3, mean ± SE. (Figs. 7B and 7D are expanded scales for IR and IR+EB 1089 \**P* < 0.05, #*P* < 0.001.).

treatment of EB 1089 + radiation (Fig. 8A, C and E). It should be noted that the low cell number for radiation alone in Figures 8B, D and F reflects sensitization to radiation through interference with the cytoprotective function of radiation-induced autophagy. Conversely, the high cell numbers for the EB 1089 + radiation combination reflects the fact that sensitization to radiation by EB 1089 was eliminated through interference with cytoprotective autophagy.

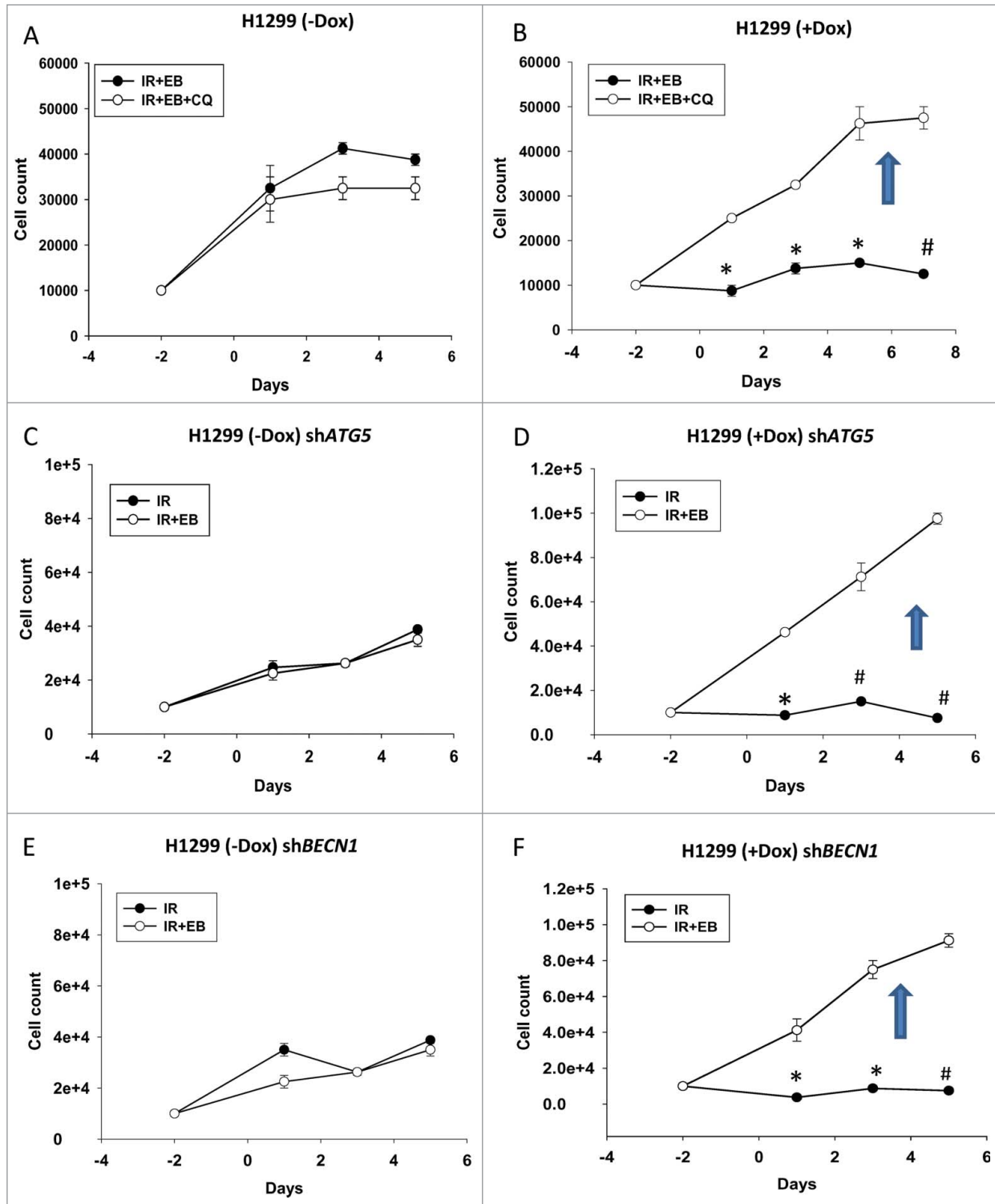
#### Studies in normal lung epithelial cells and cardiomyocytes

Drug selectivity is a critical consideration in studies related to cancer therapies; treatments should ideally be highly targeted to minimize the damage to normal cells and tissue. This is particularly true for the utilization of radiation in diseases such as non-small cell lung cancer. Consequently, studies were conducted to test for selectivity of the combination treatment of 1,25-D<sub>3</sub> with radiation in normal human bronchial epithelial

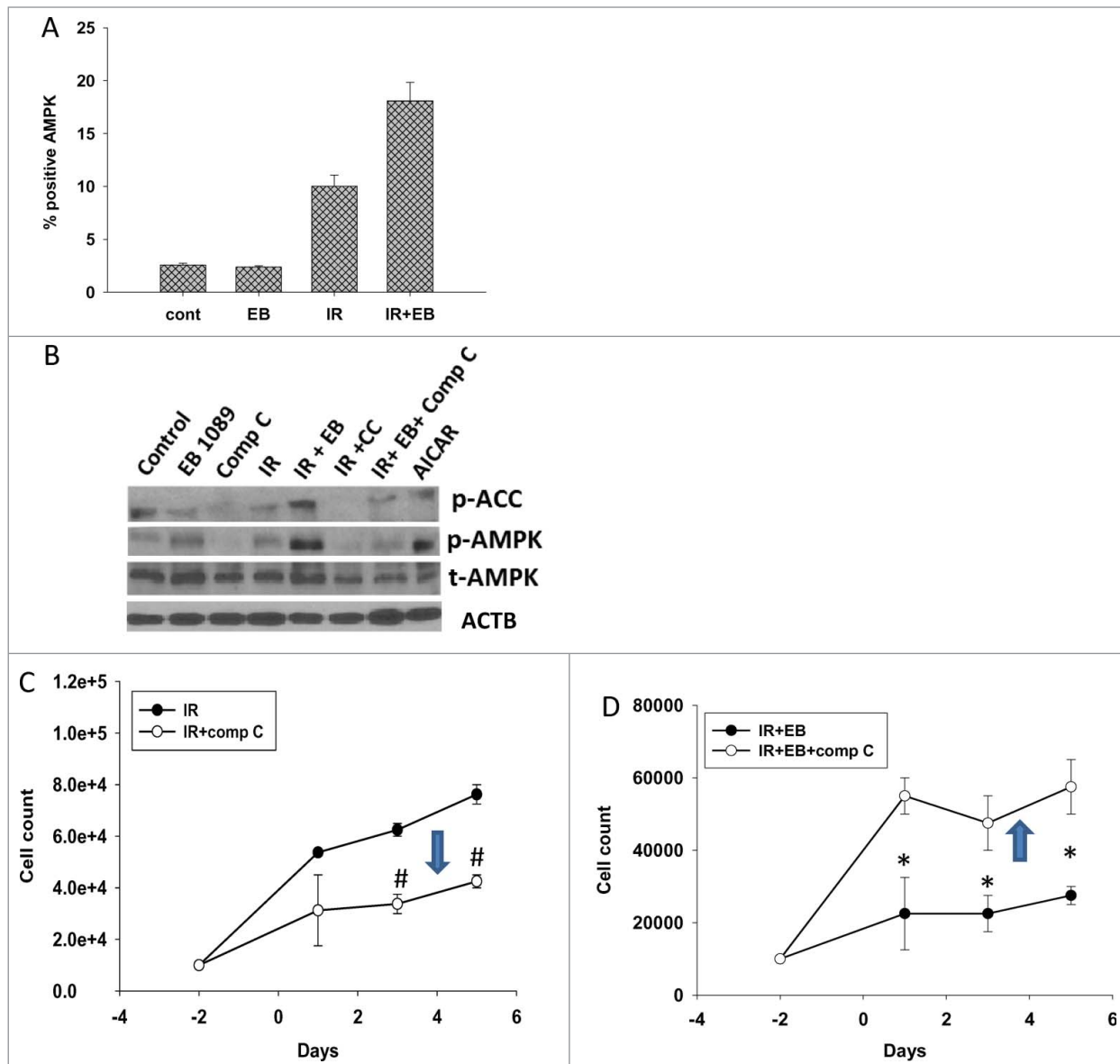
cells and cardiomyocytes in culture. As with the experiments in H460 cells, the HBEC3-KT bronchial epithelial cells were pre-treated with 100 nM EB 1089 24 h before irradiation with a single dose of 6 Gy while the H9C2 cardiomyocytes were exposed to 100 nM 1,25-D<sub>3</sub> for 72 h followed by a single dose of radiation (5 Gy). Figure S9A and B indicate that there were no significant differences between the impact of radiation alone and the combination of radiation + 1,25-D<sub>3</sub> or radiation + EB 1089 treatments in the epithelial cells or the cardiomyocytes, respectively.

#### Additional evidence for differences in the functional forms of autophagy induced by radiation alone and by radiation + EB 1089.

AMPK, an energy-sensing protein kinase, has been shown to be involved in regulation of autophagy.<sup>31,32</sup> Studies were performed to investigate changes in the phosphorylated (active)



**Figure 8.** Blocking autophagy reverses the sensitization in TP53-inducible H1299 cells. Cell viability studies indicate that inhibition of autophagy in H1299 parental *tp53*-null cells by using either. (A) chloroquine or genetic silencing of (C) *ATG5* or (E) *BECN1* did not cause any change in sensitivity of the cells. However in TP53-inducible H1299 cells blocking autophagy either by using (B) chloroquine or genetically silencing (\* $P < 0.05$ , # $P < 0.001$ ) (D) *ATG5* (\* $P < 0.05$ , # $P < 0.001$ ) or (F) *BECN1* (\* $P < 0.05$ , # $P < 0.001$ ) reversed the sensitization that was achieved with the combination treatment of EB 1089 and radiation.  $n = 3$ , mean  $\pm$  SE.



**Figure 9.** Dual roles of AMPK in cytoprotective and cytostatic autophagy. **(A)** H460 cells were exposed to 6 Gy of radiation (IR) or EB 1089 (100 nM) + radiation and phosphorylation of AMPK (Thr172) was monitored by flow cytometry at 24 h post-radiation. **(B)** Western blotting was performed to confirm successful inhibition of phosphorylation of AMPK and its downstream target ACC (acetyl-CoA carboxylase) by using compound C (inhibitor of AMPK). **(C/ D)** Effect of compound C on the temporal response to radiation (IR, 6 Gy) alone **(C)** or EB 1089 + radiation **(D)** was assessed by monitoring cell viability.  $n = 3$ , mean  $\pm$  SE, \* $P < 0.05$ , # $P < 0.001$ .

form of AMPK levels in response to radiation alone (cytoprotective autophagy) and radiation + EB 1089 (putatively cytostatic autophagy). **Figure 9A** indicates that increased phospho-AMPK levels were observed with radiation alone as well as with the combination of radiation + EB 1089 as measured by flow cytometry. After 48 h, AMPK activation by the combination treatment significantly exceeded that for radiation alone. This was confirmed by the protein gel blotting data presented in **Figure 9B** where p-AMPK levels are clearly higher for the combination treatment. A recent study conducted by Singh K et al. indicates that AMPK is activated upon irradiation of H1299 cells,<sup>33</sup> consistent with our own findings.

To potentially distinguish between the involvement of AMPK in autophagy signaling by radiation alone and radiation + EB 1089, compound C was used to inhibit AMPK. **Figure 9B** confirms that compound C interfered with the phosphorylation of AMPK and its downstream target ACC (acetyl-CoA carboxylase) induced by radiation alone as well as EB 1089 + radiation. **Figure 9D** shows that inhibition of AMPK resulted in reversal of the radiosensitizing effects of EB 1089. In dramatic contrast, treatment with compound C *enhanced* the response to radiation alone as shown in **Figure 9C**. Consequently, these findings closely parallel the outcome of the experiments presented in **Figures 4, 5 and 7**, where autophagy inhibition enhanced sensitivity to

radiation but reversed the enhancement of radiation sensitivity by EB 1089. These studies support the premise that autophagy has diametrically opposite functions in the case of radiation alone (cytoprotective) and radiation + EB 1089 (cytostatic radiosensitizing).

## Discussion

The treatment of lung cancer continues to represent a clinical challenge. Current treatments do prolong patient life but often become primarily palliative.<sup>3,4</sup> Chemo-radiation is routinely used to treat lung cancer;<sup>5-7</sup> however, the doses that can be administered are limited by toxicity to normal adjoining tissue.<sup>34</sup> Consequently, increasing the selectivity of radiation therapy would be of great benefit to lung cancer patients.

Vitamin D has been shown to have antiproliferative effects in various experimental models of cancer.<sup>11,12</sup> However, in our hands, vitamin D or EB 1089 alone, at 100 nM, showed no anti-proliferative or cytotoxic effects against the NSCLC cell lines (H460, A549, H1299 and H358) used in the current work despite high expression of VDR: this highlights that fact that the effects of the combination treatments are not merely additive but reflect a mechanistic interaction that is likely to be related to the induction of autophagy. While the combination treatment of radiation + 1,25-D<sub>3</sub> or EB 1089 resulted in cell death in breast tumor cells,<sup>18,19</sup> in the current studies we observe primarily extended growth inhibition and suppression of proliferative recovery. The combination treatment of radiation + 1,25-D<sub>3</sub> or EB 1089 did not increase either apoptosis or necrosis, similar to what we have reported previously for breast tumor cells.<sup>18,19</sup> In contrast to the studies in breast tumor cells, there was no evidence of cell death (i.e., a decline in viable cell number over time). Nevertheless, we observed unequivocal evidence for radiosensitization in clonogenic survival assays, which is consistent with a reduction in the capacity for cellular self-renewal. The observed impact on self-renewal capacity and proliferative recovery could prove to be relevant to the problem of disease recurrence in lung cancer patients.

While our initial experiments in A549 cells suggested that sensitization might be related to an increase in the extent of autophagy, subsequent experiments in H460 cells tended to rule out this possibility. Instead, our data support the relatively novel premise that the cytoprotective function of autophagy that is evident with radiation alone is converted to a cytostatic function (pronounced growth inhibition, suppression of proliferative recovery, and reduced clonogenic survival) with the combination treatment. This conclusion is supported by the observations that inhibition of autophagy, whether by pharmacological agents or genetic silencing, sensitizes the cells to radiation but protects the cells from the combination treatment. In complementary studies, inhibition of AMPK by compound C, a relatively selective inhibitor of AMPK mediated signaling,<sup>35</sup> also sensitizes the cells to radiation while protecting the cells from the combination treatment. Our argument for the conversion of one form of autophagy to the other is supported by the fact that sensitization to

radiation through autophagy inhibition appears to have been entirely eliminated from the experimental system; otherwise, we might have expected sensitization to radiation by inhibition of the protective autophagy induced by radiation alone to have masked the reversal of sensitization to EB 1089 or 1,25-D<sub>3</sub> when cytostatic autophagy was inhibited.

Recent literature has shown that TP53 is involved in regulating autophagy via the MTOR-AMPK pathway in lung cancer cells.<sup>36</sup> Our previous work established that TP53 is required for sensitization to radiation in breast tumor cells<sup>18,19</sup> and this appears to be the case for NSCLC cells as well, since H358 cells with mutant TP53 did not demonstrate any radio-sensitization by EB 1089. Furthermore, sensitization to radiation by EB 1089 could not be shown in the *tp53*-null, VDR-expressing H1299 cell line unless TP53 expression was induced under the influence of doxycycline. The requirement for functional TP53 is consistent with the apparent involvement of AMPK in autophagy as TP53 has been shown to act upstream of AMPK;<sup>36,37</sup> Additionally, Feng et al. show activation of AMPK in a TP53-dependent manner and further indicate that TP53 can bind to the  $\beta$  subunit of AMPK and lead to its activation.<sup>38,39</sup> This upregulation of AMPK has been shown to further suppress MTOR, leading to autophagy induction.<sup>38,39</sup> Future studies will be designed to address the putative relationship between TP53 and AMPK in the induction of autophagy in this experimental model as well as other potential components of the putative signaling pathway(s) such as those modulated by the ULK1/2/3/4 family and MTORC.<sup>40,41</sup> Further, as would be expected, the presence of the VDR is critical since studies conducted with TP53 wt H838 NSCLC cells, which lack VDR, also showed lack of radiosensitization with the combination treatment.

Drug selectivity and specificity are critical elements in efforts to avoid damage to normal tissue. Off-target actions of drugs can cause severe and undesired side effects. We showed that EB 1089 and 1,25-D<sub>3</sub>, respectively, do not increase radiation sensitivity in normal bronchial epithelial cells in spite of their expression of VDR<sup>42</sup> or in H9C2 cardiomyocytes which have wt TP53,<sup>43</sup> the latter being particularly relevant in cases where the heart might fall within the radiation field during the course of treatment.

The current work builds upon and extends our previous findings in breast cancer in demonstrating that 1,25-D<sub>3</sub> and the vitamin D analog EB 1089 enhance sensitivity to radiation in NSCLC cells. Again, as in previous work, sensitization is limited to NSCLC cells that express VDR and functional TP53. Furthermore, there is no apparent sensitization in normal lung epithelial cells or cardiomyocytes, consistent with previous work that indicated that sensitization did not occur in untransformed cells.<sup>44</sup> The current work also is similar to our previous findings in implicating autophagy as the mechanism underlying the radiosensitization. However, *in contrast* to the promotion of a cytotoxic form of autophagy in breast tumor cells,<sup>18,19,21</sup> we present the novel observation that the autophagy induced by radiation in combination with either 1,25-D<sub>3</sub> or EB 1089 has a cytostatic function that nevertheless translates into an enhanced reduction of clonogenic survival. Finally, in these studies, we observe the apparent



conversion of the cytoprotective form of autophagy induced by radiation alone to the cytostatic form by radiation in combination with +1,25-D<sub>3</sub> or EB 1089. While we have not directly ruled out other modes of “cell death” such as autosis or necroptosis, or methuosis,<sup>45-47</sup> in fact we do not actually observe cell death, but extensive and prolonged growth arrest. This fact, accompanied by the reversal of sensitization with autophagy inhibition, appears to support our contention that the nature of autophagy has been altered (i.e., an autophagic switch) rather than that the growth arrest induced by radiation alone has been converted to cell death.

In summary, our current studies for the first time demonstrate an enhanced responsiveness to radiation in NSCLC by 1,25-D<sub>3</sub> and EB 1089. Furthermore, this work identifies the conversion of cytoprotective autophagy induced by radiation to a novel cytostatic form of autophagy, which appears to mediate the effectiveness of this treatment combination.

## Materials and Methods

### Cell lines

The wild-type (WT) TP53 A549 cell line, VDR null H838 cell line, TP53 mutant H358 cell line, and normal bronchial epithelial cell line HBEC3-KT originally generated by the Minna laboratory<sup>48</sup> were generously provided by Dr. Charles Chalfant (Virginia Commonwealth University). H460 (WT TP53) NSCLC cells were a generous gift from Dr. Richard Moran at Virginia Commonwealth University. H460 sh*BECN1*, sh*ATG5* cells were generated as described below. H1299 *tp53*-null and TP53-inducible cells were originally developed by Dr. Constantinos Koumenis<sup>49</sup> and provided through our collaborators, Drs. Frank and Suzy Torti, (University of Connecticut).

Mission shRNA bacterial stocks for *ATG5* and *BECN1* were purchased from Sigma Aldrich (TRCN00151963 and TRCN0000299864, respectively). Lentiviruses were produced in HEK 293TN cells cotransfected using Lipofectamine (Invitrogen, 11668–019) with psPAX2 and pMD2.G packaging constructs (Addgene, 12260, 12259). Viruses shed into the media were then used to infect H460 cells. Puromycin (1 µg/ml) was used as the selection marker to enrich the infected cells.

### Cell culture and treatment

H460, A549, and H1299 cells were maintained in DMEM media supplemented with 10% (v/v) fetal bovine serum (Thermo Scientific, SH30066.03), 100 U/ml penicillin G sodium (Invitrogen, 15140–122), and 100 µg/ml streptomycin sulfate (Invitrogen, 15140–122). H838 cells were maintained in RPMI 1640 media with the same supplements. HBEC3-KT cells were maintained in Keratinocyte-SFM media (Life Technologies, 12568–010) supplemented with fetal bovine serum (provided with the kit), 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. H460 sh*ATG5* and sh*BECN1* and H1299 sh*ATG5* and sh*BECN1* cells were maintained with puromycin (1 µg/ml; Sigma, P8833) for selection. All cells were maintained at 37°C under a humidified, 5% CO<sub>2</sub> atmosphere. Except where

otherwise indicated, after equilibration post-seeding, cells were treated with single dose of 6 Gy radiation alone (using a <sup>137</sup>Cs irradiator), 100 nM 1,25-D<sub>3</sub> alone, or 100 nM EB 1089 (Tocris Bioscience, 2551, 3993) alone, or the combination of EB 1089 or 1,25-D<sub>3</sub> with 6 Gy radiation. Cells were washed free of the 1,25-D<sub>3</sub> or EB 1089 after 48 h (i.e., exposure for 24 h prior to and 24 h post irradiation). To induce TP53 expression, H1299 cells were continuously incubated with doxycycline (1 µg/ml; Sigma, 09891) throughout the experiment. Media was replenished once all the treatments were completed. H1299 sh*ATG5* and sh*BECN1* cell lines were maintained in doxycycline post transfection.

### Cell viability and clonogenic survival

Cell viability was assessed by trypan blue exclusion at various time points after the treatment. Cells were harvested using trypsin, stained with 0.4% trypan blue (Sigma, T8154), and counted using a hemocytometer under phase contrast microscopy. For clonogenic studies, cells were plated in 6-well dishes at an appropriate density. After 2 wk, the cells were washed with phosphate-buffered saline (PBS; Life Technologies, 10010023), fixed with 100% methanol, air-dried and stained with 0.1% crystal violet (Sigma, C3886). Cells in groups of 50 or more were counted as colonies and data were normalized relative to untreated controls.

### Western blotting

After the indicated treatments, cells were scraped from the culture dishes, collected, and lysed using M-PER mammalian protein extraction reagent (Thermo Scientific, 78505) containing protease and phosphatase inhibitors (Sigma Aldrich, P8340, P5726). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, 500–0205r). Total protein was then diluted in SDS sample buffer and dry boiled for 10 min. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blocked in 5% milk, 1x PBS, 0.1% Tween 20 (Fisher, BP337) for 1 h. Primary antibodies used at a 1:1000 dilution with overnight incubation were SQSTM1/p62 (BD Biosciences, 610497), *ATG5* (Cell Signaling Technology, 2630), *BECN1* (BD Biosciences, 612112), VDR (Santa Cruz Biotechnology, sc1008), LC3B-I/LC3B-II (Cell Signaling Technology, 3868), phospho-AMPK (Cell Signaling Technology, 2531), total-AMPK (Cell Signaling Technology, 2795s), phospho-ACAC/acetyl-CoA carboxylase (p-ACC; Cell signaling Technology, 3661s), TP53 (BD Biosciences, 554157), ACTB/β-actin (Sigma, A5316). The membrane was then incubated with secondary antibody of either horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:10000; GE Healthcare, NA434) or goat anti-mouse (1:10000; GE Healthcare, NA931) for 1 h, followed by extensive washing with Tween-PBS (PBS with 0.1% Tween 20). Blots were developed using Pierce enhanced chemiluminescence reagents and Bio-Max film (Phenix Research Products, F-BX57).

### Detection and quantification of acidic vesicles with acridine orange staining

Cells were plated in 6-well culture dishes, stained with acridine orange (Sigma, A6014) at a final dilution of 1:10,000 in respective culture media and allowed to incubate at 37°C for 10 min. After 2 washes with PBS, cells were imaged under an inverted fluorescence microscope (Olympus, Tokyo, Japan). All images were taken at the same magnification. For quantification of autophagic vesicles (AVOs), cells were trypsinized, harvested, and washed with PBS at approximately  $1 \times 10^8$  cells/ml. Pellet fractions were resuspended in PBS, stained with a 1:10,000 dilution of acridine orange for 10 min and analyzed by BD FACSCanto II (Virginia Commonwealth University) using BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core Facility. A minimum of 10,000 cells within the gated region was analyzed. Data collected was further analyzed using FCS Express 4 Flow research edition.

### Detection and quantification of senescent cells

The method described by Dimri et al.<sup>50</sup> was used for detection of GLB-positive cells. After treatment, cells were washed with PBS and fixed with 2 % formaldehyde/0.2% glutaraldehyde/PBS for 5 to 8 min. Cells were again washed with PBS and stained with 5-bromo-4-chloro-3-inolyl- $\beta$ -D-galactopyranoside (Fisher, R0404) in dimethylformamide (20 mg/ml), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl<sub>2</sub> and incubated at 37°C for 24 h. After incubation, the cells were washed twice with PBS and visualized using an Olympus inverted microscope. All images were taken at the same magnification. For quantification of SA-GLB/ $\beta$ -galactosidase staining, cells were treated as above and analyzed using a fluorescent GLB activity marker C<sub>12</sub>FDG (Life Technologies, D2893). The protocol was adapted from Debacq-Chainiaux et al.<sup>51</sup>

### RFP-LC3 and GFP-LC3 redistribution

The RFP-LC3 construct was a generous gift from Dr. Keith Miskimins, Sanford Research, University of South Dakota. H460 cells were transfected with RFP-LC3 using lipofectamine. A549 cells were transfected with GFP-LC3 (Addgene, 22405) using lipofectamine (Invitrogen, 11668–019). Cells were treated as described above, fixed with 3% paraformaldehyde, and visualized using a Leica Confocal laser-scanning microscope (Virginia Commonwealth University). Cells were counterstained with DAPI to stain and visualize the nucleus.

### FACS analysis for ANXA5 and propidium Iodide (PI)-positive cells to assess apoptosis

After treatments, cells were collected, washed with PBS, and resuspended in 500  $\mu$ l of 1x Binding Buffer (ANXA5-FITC apoptosis detection kit; BD Biosciences, 556547) with 5  $\mu$ l of ANXA5-FITC, and 5  $\mu$ l of propidium iodide. Samples were gently mixed and incubated at room temperature in the dark for 15 min. The number of cells with increased ANXA5 and PtdIns

staining was assessed by flow cytometry and analyzed by BD FACSCanto II using FACSDiva software. A minimum of 10,000 cells within the gates region was analyzed.

### FACS analysis for cell cycle distribution

The protocol described by Menon VR et al.<sup>52</sup> was used to assess cell cycle distribution. H460 control, H460 sh*ATG5* and sh*BECN1* cells were seeded in 100-mm tissue culture plates at different densities based on the time points for collection. After 24 h, cells were exposed either to radiation alone, EB 1089 alone, or the combination of EB 1089 and radiation. At indicated time points,  $1 \times 10^6$  cells were suspended in 1 ml PI solution (Sigma, P4864) with added RNase A (Sigma, R5125) at a final concentration of 0.2 mg/ml. Cells were maintained in the dark at 4°C overnight and analyzed the next day to measure the DNA content by flow cytometry.

### Assessment of AMPK phosphorylation and H2AFX/ $\gamma$ -H2AFX formation

After the indicated treatments, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized with 100% cold methanol for 30 min and incubated with blocking buffer (4% BSA [Fisher, NC9857891] in PBS) for 10 min. Cells were incubated with a 1:50 dilution of primary antibody for 1 h, washed with PBS and incubated with a fluorochrome-conjugated secondary antibody (1:100 dilution) for 30 min in the dark. After the second incubation, cells were washed with blocking buffer and resuspended in 400  $\mu$ l PBS. Cells were analyzed using FACS with a minimum of 10,000 cells for each sample.<sup>53</sup> Primary antibodies used were p-PRKAA1/PRKAA2 (AMPK  $\alpha$ 1/ $\alpha$ 2 Thr172; Cell signaling Technology, 2531), and anti-p- $\gamma$ -H2AFX ( $\gamma$ -H2AX Ser139) (EMD Millipore, 07–627).

### Statistical analysis

Statistical differences were determined by StatView statistical software (SAS Institute, Cary, NC). The data were expressed as means  $\pm$  SE (as standard error of the mean). Comparisons were made using one-way analysis of variance (ANOVA) followed by Turkey-Kramer post hoc test, and P values  $\leq$  0.05 were taken as statistically significant.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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